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(57) Abstract

The invention relates to a novel mutant of tissue factor pathway inhibitor (TFPI) protein and its corresponding DNA sequence. The mutant can be found in humans who show or may show an increased risk of thrombotic diseases. By screening samples of human blood for said DNA or fragments of it, it is possible to predict a disposition of thrombotic disorders by which prophylactic application or measures can be initiated.

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A Mutant of Tissue Factor Pathway Inhibitor, DNA Sequence and Use for Detecting Thrombotic Disorders

The invention relates to a novel mutant of tissue factor pathway inhibitor (TFPI) protein and its corresponding DNA sequence. The mutant can be found in humans who show or may show an increased risk of thrombotic diseases. The DNA sequence according to this invention differs from the known TFPI coding DNA by a single nucleotide polymorphism which results in a change of one amino acid position within the known TFPI protein. By screening samples of human blood for said DNA or fragments of it, it is possible to predict a dispostion of thrombotic disorders by which prophylactic application or measures can be initiated.

Tissue factor pathway inhibitor (TFPI) is an important regulator in the extrinsic blood coagulation pathway. Although the regulatory biochemical role of TFPI is evident, the clinical significance of this proteinase inhibitor remains to be elucidated. The definition of a clinical TFPI deficiency.seems to be more complex than that of other coagulation inhibitors because the activity and concentration of circulating TFPI can not be considered a true measure of *in vivo* levels. Its determination in plasma samples by immunological methods or functional assays has been shown to be inadequate in the detection of a clinical deficiency.

TFPI is a single chain glycoprotein present in plasma in trace amounts. It was previously known as extrinsic pathway inhibitor, or lipoprotein associated coagulation inhibitor. TFPI belongs to the class of Kunitz-type proteinase inhibitors, and the mature protein contains an acidic amino-terminal end followed by three Kunitz-type inhibitory domains and a basic carboxy-terminal end. The cDNA coding for TFPI was cloned and characterized by Wun et al. (J. Biol. Chem. (1988), 263). The mature molecule consists of 276 amino acid residues, including 18 cysteins (see Seq. Id. No. 1, 2), all involved in disulfide bonds, and contains three potential N-linked glycosylation sites. The molecular weight of the polypetide backbone is about 32 kDA; the protein present in plasma runs, however, on SDS-PAGE with an apparent molecular weight of about 42 kDa,

presumably due to glycosylation.

The multivalent protease inhibitor is an important regulator of the extrinsic pathway of blood coagulation through its ability to interact with the blood coagulation factor VIIa / tissue factor complex and the activated factor X via its *Kunitz*-type domains δ1 and δ2 *(see: Girard, T.J. et al. , Nature **338**, 518-520 (1989); Broze, G.J.Jr. et al.; Blood **71**, 335 (1984); Rapaport, S.I. & Rao, L.V.M., Thrombosis and Haemostasis **74**, 7-17 (1995); Broze, G.J.Jr. , Haemostaseologie **17**, 73-77 (1997))*. There is also evidence that infusion of recombinant TFPI may protect against disseminated intravascular coagulation induced by TF or E. coli to protect against venous thrombosis and to prevent rethrombosis after successful thrombolysis in arterial thrombosis. The intravascular distribution of TFPI is complex. The mature human tissue factor pathway inhibitor protein is mainly synthesized by the vascular endothelium *(Bajaj, M.S. et al. Proc. Natl. Acad. Sci. USA **88**, 8869, (1990))*. It has also been detected in at least four intravascular pools: bound to the endothelial cell surface, associated with lipoproteins, carrier-free within the plasma, and sequestered in platelets *(Sandset, P.M. & Abildgaard, U., Haemostasis **21**, 219 (1991))*.

A review of the regulation and role of TFPI within the extrinsic pathway system is given by Petersen at al. *(Thrombosis Research, 79, 1-47 (1995))*.

TFPI plays such an important role in the inhibition of the extrinsic pathway that TFPI deficiencies due to mutations in the TFPI gene should enhance the activity of the prothrombinase complex. This increases the thrombin generation and consequently the risk of venous thrombosis. Such a diminished inhibition of thrombin generation is already well known in inherited coagulation inhibitor defects that predispose to thrombosis including deficiency of antithrombin III, protein C and protein S *(Dahlbäck, B. Blood **85**, 607-614 (1995)*). The most prevalent inherited abnormality which is known to lead to venous thrombosis is the resistance to activated protein C caused by a single point mutation in the factor V gene *(Bertina, R.M. et al., Nature **369**, 64-67 (1994))*.

Thus it was an object of the present invention to screen genomic DNA samples of human normal blood donors and thrombotic patients for alterations in the TFPI gene to assess the influence of a modified TFPI in venous thromboembolic

diseases.

Surprisingly, a single nucleotide substitution (C→T) in exon 7 of the TFPI gene could be detected, leading to a proline to leucine exchange at amino acid position 151 of the mature protein (see Seq. Id. No. 3, 4). Investigating the statistical significancy of this modification of TFPI it was found that said modification can be linked to a relative risk to general thrombotic disorders. However, it should be pointed out that said modification seems also to occur in human individuals showing evidently - by comparing their family health history - no increased risk to thrombotic events. Nevertheless, the finding of modified TFPI protein / DNA in blood samples of a human individual may provide an important hint for a possible disposition of said individual for thrombotic diseases. Therefore, prophylactic measures can be taken in order to prevent said diseases.

According to this invention the term "thrombotic disorders" used above and below includes all known diseases or malfunctions which can be related directly or indirectly to a permanent or temporary abnormal or pathological blood coagulation, e.g. venous thrombosis.

The modified TFPI protein or related DNA can be used as diagnostic marker to detect such a possible risk to thrombotic disorders in a patient.

Thus, it is an object of this invention to provide a DNA sequence coding for a mutant of tissue factor pathway inhibitor (TFPI) protein, wherein a proline residue at position 151 of the mature peptide (calculated from the N-terminus) is replaced by a leucine residue.

It is a further object of the invention to provide a DNA sequence comprising a DNA sequence (a) coding for a signal peptide followed by a DNA sequence (b) coding for a mutant of TFPI protein, wherein a cytosine at position 536, calculated from the start codon of the sequence (a) is replaced by a tyrosine to form a CTG codon instead of a CCG codon within the coding region of sequence (b).

Furthermore, it is an object of the invention to provide a diagnostic method to detect a disposition for thromboembolic diseases in humans by screening

genomic DNA samples of human blood for a DNA sequence defined above or in the claims.

Especially, the invention relates to a diagnostic method using polymerase chain reaction (PCR) and restriction analysis by extracting total DNA from human blood samples amplifying exon 7 of the TFPI gene (from position 536 to 628) and its previous flanking intronic region by means of suitable primers, treating the PCR products with a restriction enzyme having the recognition site ACTGG or CAGTG, and detecting the fragment length after restriction analysis or isolating and detecting the DNA defined above and in the claims.

Suitable primers according to this inventions are primers or DNA fragments which may hybridize with the corresponding regions of exon 7 of the TFPI gene, intronic flanking regions included. Therefore, as preferred embodiments, it is an object of this invention to provide a diagnostic method defined above and in the claims using the following primers (Seq. Id. No. 6 , 8):

5' - TCTATTTTAATTGGCTGTAT - 3' and

5' - GCATGATAATAGTTTCCTGG - 3'.

The single nucleotide polymorphism modification (C → T, CCG → CTG) in the TFPI gene at position 536 creates a new cleavage site within this region of the gene, which is not present in the original gene. This is very advantageous and can be preferably used for short evidence of such modified DNA in blood samples and, as consequence, of the above-mentioned disposition for thrombotic disorders. The recognition site created by said nucleotide exchange is ACTGG or CAGTG (Seq. ID. No. 5, 7). Therefore, all restriction enzymes which can recognize this cleavage site are suitable to carry out the diagnostic method of the invention.

Known and suitable restriction enzymes are, for example, Bse1I, BseNI, BsrI, BsrSI, BscH1, Bst11I, BsoHI, Tsp1I, and TspRI. The preferred resriction enzyme of the invention is BseNI.

Finally, it is an object of the present invention to provide the novel mutant of tissue factor pathway inhibitor (TFPI) protein wherein a proline residue at position 151 of the mature peptide (calculated from the N-terminus, without signal peptide

sequence) is replaced by a leucine residue.

According to this invention it was proved the thesis that genetic variations in the TFPI gene contribute to the occurrence of hitherto unexplained cases of thrombophilia in about half of the afflicted patients. In a first screening experiment 50 unrelated individuals with a thrombotic history were investigated, who were selected to determine the genetic basis of their thrombosis (27 of them where shown to be carriers of the factor V Leiden mutation). While scanning all coding exons of the TFPI gene and the adjacent 5' and 3' intronic regions by PCR-SSCP analysis, an abnormal pattern suggesting the presence of a genetic variation, was observed in a PCR fragment from exon 7 (Fig. 1a). DNA sequencing of the fragment showing the abnormal SSCP banding pattern revealed a single heterozygous C to T mutation at nucleotide position 1 of exon 7, changing the codon CCG^{151} to CTG^{151} resulting in a Pro^{151} to Leu^{151} exchange in the amino acid of the mature protein (Fig. 1b, c; Fig. 2) *(see also: van der Logt et al., Biochemistry **30**, 1571-1577 (1991); Girard, T.J. et al.; J. Biol. Chem. **266**, 5036-5041 (1991))*.

The 536C→T transition is associated with the creation of a new recognition site for the restriction enzyme *Bse*NI, providing a rapid means of screening further individuals for this mutation by PCR and restriction fragment length polymorphism analysis (PCR-RFLP). The primers designed for the amplification of exon 7 and the 5' and 3' flanking intronic regions were used to generate a 170 bp PCR fragment. If the nucleotide C is present at position 536, the 170 bp DNA fragment is not digestible with *Bse*NI. However, if the nucleotide T is present at this position a 27 bp and a 143 bp restriction fragment is generated. Using this method a second set of patients was sreened with venous thrombosis (n=324) of whom 30.2% carried the factor V Leiden mutation. Another individual heterozygous for the TFPI mutation was detected. Homozygous carriers of this mutation were not found.

In order to estimate the prevalence of the 536C→T exchange in the general population, 2480 randomly chosen unrelated blood donors (age 18 - 60 years) were investigated by PCR-RFLP analysis.

To detect a greater number of subjects with the trait, which would allow a more precise estimation of the relative risk of those individuals to develop venous thrombosis, family members of the heterozygote blood donors and patients were investigated. In total 13 heterozygote individuals within 6 different families were found. Two of them suffered from deep vein thrombosis.

All subjects carrying the TFPI mutation were investigated for the presence of other genetic defects of clotting proteins (factor V Leiden, prothrombin 20210G→A mutation, deficiency of protein C, protein S and antithrombin III) in order to exclude the contribution of these disorders to the thromboembolic findings. The two individuals who showed the TFPI trait and suffered from venous thrombosis had none of the additionally examined genetic disorders. Although the TFPI mutation was observed together with either the factor V Leiden mutation or the prothrombin mutation (20210G→A) in 4 members of one family, none of them had a history of thromboembolic diseases.

It is generally accepted that direct evidence for an important regulatory role of an inhibitory clotting protein requires the detection of low levels of the circulating inhibitor associated with venous thrombosis. Therefore, the TFPI activity was measured by a functional assay and the protein concentration by an immunological assay in plasma of all individuals showing the TFPI mutation. Compared with the control group (blood donors without TFPI mutation) no statistically significant differences were detected. However, existing small differences might not have been recognized, due to the low number of cases investigated so far.

Furthermore, for an interpretation of plasma TFPI levels it must be taken into consideration that compared with other coagulation inhibitors such as antithrombin III and protein C which circulate mainly as free molecules, circulating TFPI may not reflect the true measure of the total *in vivo* TFPI quantity. Therefore, determination of TFPI activity or protein concentration in plasma samples has been shown to be an inadequate approach to detect clinical TFPI deficiencies. The intravascular distribution of TFPI is more complex. A major pool of TFPI (about 50-80% of the total intravascular pool) is normaly bound to the

endothelium, but may be released into the circulation following injection of heparin. It is also a known fact that more than 80% of the circulating TFPI is in a complex form with lipoprotein, preferentially with low density lipoprotein. However, whether this fraction of lipoprotein-associated TFPI remains active as a clotting inhibitor is still unknown. The plasma TFPI level correlates with the lipoprotein concentration, and it has been shown that patients with an inherited abetalipoproteinemia have decreased TFPI plasma concentrations but do not suffer from venous thrombosis. Therefore, the questions arises whether a deficiency of total intravascular TFPI can be detected by any of the tests used so far. (*Sandset, P.M. & Bendz, B. Thrombosis and Haemostasis 78, 467-470 (1997)*). A genetic abnormality of TFPI which promotes the development of venous thrombosis may affect different functions of the protein, such as secretion by endothelial cells, binding to the endothelial membrane, proteolysis in the vascular space, and association with lipoproteins.

So far, 13 individuals with the TFPI mutation were identified. Two of them had a history of venous thrombosis. Although the number of subjects is rather small to evaluate the risk of thrombophilia, the prevalence of venous thromboembolism in this group was compared with the prevalence in 466 blood donors who were thoroughly questioned about a possible history of venous thrombosis. 10 cases were found in this group. Statistical analysis showed a probability of 94.5% for the hypothesis that the presence of the TFPI trait is linked to thrombophilia (p=0.055; odds ratio: 5.9; 95% confidence interval: 1.0-36.5). Thus, this question remains open and can only be answered when more subjects with the TFPI mutation are available for statistical evaluation.

Description of the figures:

Fig. 1: PCR-SSCP and sequence change for the proline to leucine substitution in a TFPI variant.

a) Single strand conformation polymorphism in exon 7 of the TFPI gene in a thrombotic patient (lane C) and nonthrombotic controls (lanes A, B, D). The arrow in lane C indicates an additional band only observed in heterozygous variants.
b) Sequencing profile of the sense strand of the PCR products obtained from total DNA from the heterozygous person identified by SSCP. The C→T transition in

the nucleotide sequence, indicated by two peaks at the same position in the heterozygous probe is marked by an arrow.

c) The C→T transition at nucleotide position 1 of exon 7 results in a CCG → CTG change in the sense strand and leads to a proline to leucine substitution at position 151 of the TFPI protein.

Fig. 2: Proposed secondary structure of the tissue factor pathway inhibitor and the position of the amino acid exchange. The mature protein consists of 276 amino acids, forming three tandem *Kunitz*-type proteinase inhibitory domains, two connecting chains, an acidic N-terminus with negatively charged amino acids (-) and a basic carboxy-terminal end, containing a cluster of positively charged amino acids (+). *Kunitz*-domain δ1 has been identified as binding site for the factor VIIa / tissue factor complex, while domain δ2 binds activated factor Xa *(Wesselschmidt, R. et al., Blood **79**, 2004-2010 (1992))*. Whether *Kunitz*-type domain δ3 binds and inhibits a specific protease is unknown. The basic carboxy-terminal end is the predicted binding site to glycosaminoglycans at the endothelial cell surface and contributes to heparin binding *(Enjyoji, K.-I. et al., Biochemistry **34**, 5725-5735 (1995))*. Truncated forms of TFPI are tightly bound to low density lipoproteins and lack the distal portion of the full-length molecule including *Kunitz*-type domain δ3 *(Broze, G.J.Jr. et al., Blood Coag. Fibrinol. **5**, 551-559 (1994))*. Asn^{117} and Asn^{167} are N-glycosylated, Ser^{174} and Thr^{175} are O-glycosylated (*) *(Nakahara, T.M. et al. Biochemistry **35**, 6450-6459 (1996))*. The Pro^{151} to Leu change is located near the *Kunitz*-type inhibitor domain δ2 within the second connecting chain (magnification).

The following examples describe the invention in more detail without restricting it.

Example 1:

Polymerase chain reaction (PCR). 374 unrelated patients with a definite history of deep vein thrombosis and 2480 blood donors as a control group were investigated in this study. Total DNA of all subjects was extracted from whole blood using the QIAamp Blood Kit (QIAGEN, Hilden, Germany). TFPI-exon 7 and its 5' and 3' flanking intronic regions were specifically amplified from genomic DNA by PCR *(Saiki, R.K. et al., Science **239**, 487-491 (1988))*. The primers for

the amplification reaction (TFPI Ex7F 5'-TCTATTTTAATTGGCTGTAT-3', TFPI Ex7R 5'-GCATGATAATAGTTTCCTGG-3') were derived from the genomic sequence of the TFPI gene *(van der Logt et al., Biochemistry **30**, 1571-1577 (1991))*. The standard PCR included 0.1 - 1 µg of genomic DNA, 300 nM of each primer, 200 µM of each deoxynucleotide triphosphate, GeneAmp 10x PCR Buffer and 2.5 units of AmpliTaq DNA Polymerase (Perkin-Elmer Corporation, Foster City, CA) in a final volume of 50 µl. After mixing, 1 drop of mineral oil was added to each tube to prevent evaporation. Thermal cycling conditions included initial denaturation at 94°C for 3 minutes, followed by 40 cycles of denaturating at 94°C for 30 sec, annealing at 48°C for 45 sec and extending at 72°C for 45 sec. The PCR products were electrophoresed on a neutral 0.8% agarose gel and stained with ethidium bromide for inspection.

Example 2:

Single Strand Conformation Polymorphism (SSCP). Sequence variations within the amplified DNA were detected by single strand conformation polymorphism following the PCR. For SSCP analysis the amplified DNA fragments containing the coding region of exon 7 were purified using the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany). Following this procedure, the fragments were diluted 1 : 6 in distilled water, heated for 10 minutes at 98°C, and then quenched on ice to achieve almost complete denaturation. Electrophoresis was performed using the PhastSystem electrophoresis unit of Amersham Pharmacia Biotech (Uppsala, Sweden). Strand separation was obtained with a 12.5% Homogeneous PhastGel at a temperature of 12°C. Running conditions were: (i) Pre-run: 400 V, 5.0 mA, 1.0 W, 12°C, 60 Vh; (ii) Sample application: 25 V, 5.0 mA, 1.0 W, 12°C; 2 Vh; (iii) Main-run: 200 V, 5.0 mA, 1.0 W; 12°C, 220 Vh. Gels were silver-stained in the coloration unit of the device, following the method described by Bassam *et al. (Bassam, B.J. et al, Analyt. Biochem. **196**, 80-83 (1991))*.

Example 3:

DNA-Sequence analysis of SSCP variants. The purified PCR fragments showing differences in the SSCP banding patterns were used as template for forward and reverse cycle sequencing reactions with the primers described above. The fragments were sequenced using the Applied Biosystems

Incorporated (ABI) protocol for TAQ cycle sequencing with dye terminators and an automated ABI PRISM 377 DNA Sequencer (Applied Biosystems, Weiterstadt, Germany).

Example 4:

PCR - Restriction Fragment Length Polymorphism (RFLP) analysis for the determination of the TFPI 536C → T mutation. PCR was carried out as described above. The PCR product was controlled on a 0.8% agarose gel. 6 µl of the PCR product were then incubated for 1.5 h with 5U of *Bse*NI (MBI Fermentas, St. Leon-Rot, Germany) at 65°C in a final volume of 20 µl without further purification. Following this, the samples were loaded onto a 2.5% agarose gel, ethidium bromide stained, and analyzed under UV-light.

Example 5:

PCR-RFLP analysis for the determination of the Factor V 1691 G→A mutation. The PCR-RFLP analysis of the factor V Leiden mutation was carried out as previously described by Beauchamp *et al.*, using the primers Fv3 and Fv6 *(Beauchamp, N.J. et al., Brit. J. Haematol. **88**, 219-222 (1994)).*

Example 6:

PCR-mediated site-directed mutagenesis for the determination of the 20210 G→A transition in the prothrombin gene. The G to A transition at position 20210 in the prothrombin gene was determined after amplification with primer PTHF 5'-CGCCTGAAGAAGTGGATACAGA-3' and PTHR 5'-ATAGCACTGGGAGCATTGAA GC-3'. The latter was designed with a C to A substitution at position 20214 to create a restriction site for *Hin*dIII (MBI Fermentas, St. Leon-Rot, Germany) when the G to A transition at position 20210 is present in the prothrombin gene *(Poort, R.S. et al., Blood **88**, 3968-3703 (1996)).* Restriction analysis and gel electrophoresis were carried out as described above under reaction conditions recommended by *Hin*dIII.

Example 7:

Measurement of TFPI concentration and activity in plasma samples. Total and full-length forms of TFPI, complexes with tissue factor (TF) and factor VIIa as

well as binary complexes with factor Xa and quaternary complexes with TF, factor VIIa and factor Xa were quantified in plasma samples obtained from individuals heterozygous for the TFPI mutation, unaffected members of their families and blood donors, using the IMUBIND Total and Truncated TFPI ELISA Kit (American Diagnostica Inc., Greenwich, CT) according to the manufacturer's instructions. The same plasma samples were used to determine the activity of predominantly free TFPI with the ACTICHROME TFPI Activity Assay from American Diagnostica Inc. (Greenwich, CT).

Example 8:

Determination of protein C and antithrombin III concentration in plasma samples. The protein C and AT III concentration in plasma samples was measured using the DADE BEHRING (Liederbach, Germany) Protein C and the Antithrombin III Chromogenic Assays according to the manufacturer's instructions.

Example 9:

Determination of protein S activity in plasma samples. The activity of the protein C cofactor was determined with the Protein S Clotting Test of Boehringer Mannheim (Mannheim, Germany). The clotting times were measured with a ball coagulometer (Amelung, Lemgo, Germany).

Example 10:

Determination of high and low density lipoprotein. To exclude plasma samples from patients with abnormal lipoprotein concentration due to association of TFPI to low density lipoprotein we determined the concentration of high and low density lipoprotein by a commercially available turbidimetric assay (Boehringer Mannheim, Mannheim, Germany).

Example 11:

Statistical analysis. The statistical analysis was performed using Chisquare test and linear logistical regression analysis for sex and age dependent calculations with the Statistical Analysis System (SAS) program, version 6.12 and Student's t-test. For statistical analysis the patients with thrombotic history were matched to the control group according to age and sex.

Patent Claims

1. A DNA sequence coding for a mutant of tissue factor pathway inhibitor (TFPI) protein wherein a proline residue at position 151 of the mature peptide (calculated from the N-terminus, without signal peptide) is replaced by a leucine residue.

2. A DNA sequence comprising a DNA sequence (a) coding for a signal peptide followed by a DNA sequence (b) coding for a mutant of TFPI protein, wherein a cytosine at position 536, calculated from the start codon of the sequence (a) is replaced by a tyrosine to form a CTG codon instead of a CCG codon within the coding region of sequence (b).

3. A DNA sequence comprising the DNA sequence of Seq. Id. No. 3.

4. Use of a DNA sequence of any of the claims 1 to 3 for the *in vitro* diagnosis of thrombotic disorders.

5. Use according to claim 4 for the *in vitro* diagnosis of a disposition for venous thromboembolic diseases in humans.

6. A diagnostic method to detect a disposition for thromboembolic diseases in humans by screening genomic DNA samples of human blood for a DNA sequence according to any of claims 1 to 3.

7. A diagnostic method of claim 6 using polymerase chain reaction (PCR) and restriction analysis by extracting total DNA from human blood samples amplifying exon 7 of the TFPI gene and its previous flanking intronic region by means of suitable primers, treating the PCR products with a restriction enzyme having the recognition site ACTGG or CAGTG, and detecting the fragment length after restriction analysis or isolating and detecting the DNA sequence of any of claims 1 to 3.

8. A diagnostic method of claim 7, wherein the following primers are used:
   (a) 5' - TCTATTTTAATTGGCTGTAT - 3' and
   (b) 5' - GCATGATAATAGTTTCCTGG - 3'.

9. A diagnostic method of any of claims 6 to 8, wherein the restriction enzyme is BseNI.

10. A mutant of tissue factor pathway inhibitor (TFPI) protein wherein a proline residue at position 151 of the mature peptide (calculated from the N-terminus, without signal peptide) is replaced by a leucine residue.

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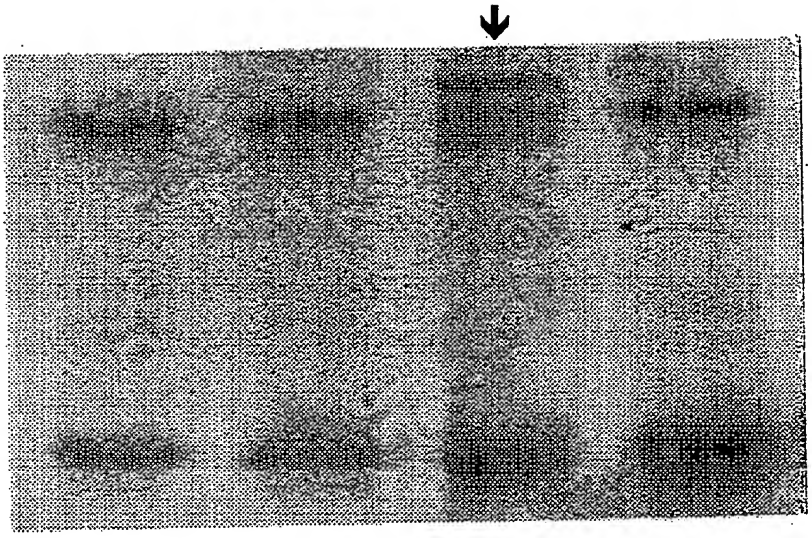

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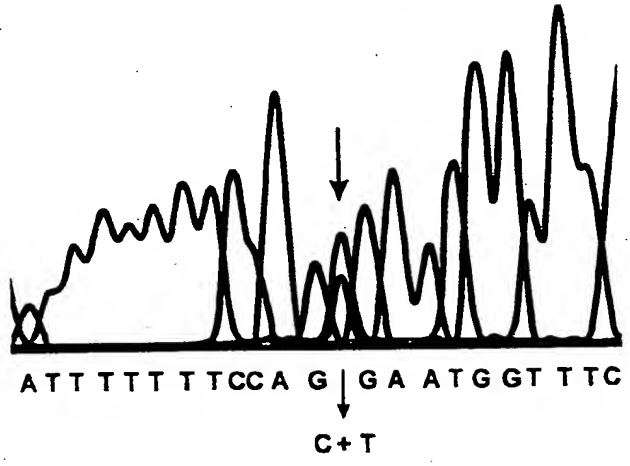
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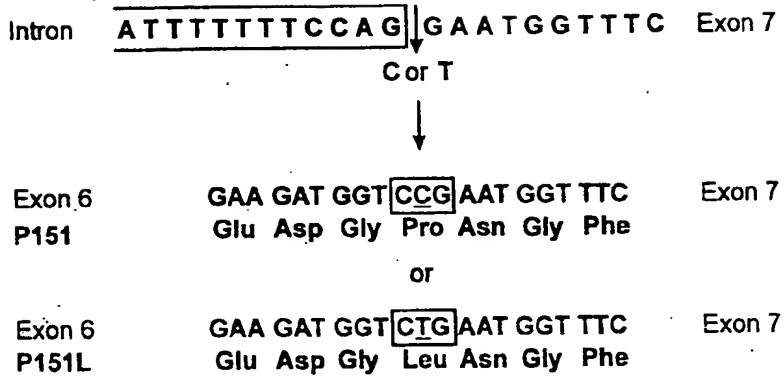
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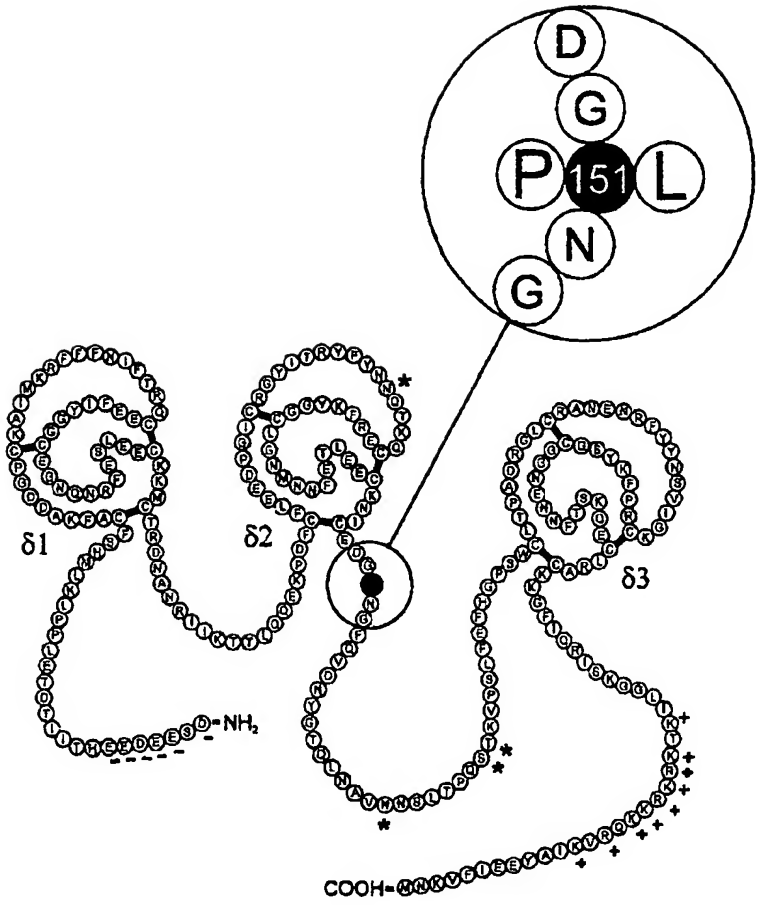
Glu Asp Gly Pro Asn Gly Phe
Exon 7
or
Exon 6
P151L
GAA GAT GGT CTG AAT GGT TTC
Glu Asp Gly Leu Asn Gly Phe
Exon 7

Fig. 1

FIG. 2



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Leu Leu Leu Asn Leu Ala Pro Ala Pro Leu Asn Ala Asp Ser Glu Glu
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gat gaa gaa cac aca att atc aca gat acg gat ttg cca cca ctg aaa   144
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Leu Met His Ser Phe Cys Ala Phe Lys Ala Asp Asp Gly Pro Cys Lys
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ctg gaa gag tgc aaa aaa atg tgt aca aga gat aat gca aac agg att   336
Leu Glu Glu Cys Lys Lys Met Cys Thr Arg Asp Asn Ala Asn Arg Ile
      70                  75                  80

ata aag aca aca ttg caa caa gaa aag cca gat ttc tgc ttt ttg gaa   384
Ile Lys Thr Thr Leu Gln Gln Glu Lys Pro Asp Phe Cys Phe Leu Glu
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gaa gat cct gga ata tgt cga ggt tat att acc agg tat ttt tat aac   432
Glu Asp Pro Gly Ile Cys Arg Gly Tyr Ile Thr Arg Tyr Phe Tyr Asn
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gct gtg aat aac tcc ctg act ccg caa tca acc aag gtt ccc agc ctt   624
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ttt act tcc aaa caa gaa tgt ctg agg gca tgt aaa aaa ggt ttc atc   816
Phe Thr Ser Lys Gln Glu Cys Leu Arg Ala Cys Lys Lys Gly Phe Ile
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caa aga ata tca aaa gga ggc cta att aaa acc aaa aga aaa aga aag   864
Gln Arg Ile Ser Lys Gly Gly Leu Ile Lys Thr Lys Arg Lys Arg Lys
245                 250                 255                 260

aag cag aga gtg aaa ata gca tat gaa gaa att ttt gtt aaa aat atg   912
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INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/EP 99/06054

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/81 C12N15/52 C12N15/10 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 93 14122 A (NOVONORDISK AS) 22 July 1993 (1993-07-22) abstract page 4, line 12 -page 5, line 30 page 7, line 9-20 ---	1,10
A	WO 96 04378 A (CHIRON CORP) 15 February 1996 (1996-02-15) abstract page 4, line 1-12 page 5, line 10 -page 7, line 15 page 7, line 31 -page 9, line 21 --- -/--	1,4,10

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 December 1999	20/12/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mateo Rosell, A.M.

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INTERNATIONAL SEARCH REPORT

Inter ›nal Application No
PCT/EP 99/06054

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	GIRARD T J ET AL., : "Functional significance of the Kunitz-type inhibitory domains of lipoprotein-associated coagulation inhibitor" NATURE, vol. 338, 1989, pages 518-520, XP000857349 cited in the application the whole document	1
A	SANDSET P M: "Tissue factor pathway inhibitor (TFPI): An update." HAEMOSTASIS, vol. 26 (SUPPL. 4), 1996, page 154-165 XP000862822 the whole document	1
P,X	KLEESIEK K ET AL.,: " A first mutation in the human tissue factor pathway inhibitor gene encoding 'P151L!TFPI" BLOOD, vol. 92 (10), 15 November 1998 (1998-11-15), page 3976-7 XP000856946 the whole document	1-10
P,X	KLEESIEK K ET AL., : "The 536CfwdarwT transition in the human tissue factor pathway inhibitor (TFPI) gene is statistically associated with a higher risk for venous thrombosis." THROMBOSIS AND HAEMOSTASIS , vol. 82 (1), July 1999 (1999-07), page 1-5 XP000856948 the whole document	1-10
P,A	MIYATA T ET AL., : "C-399T polymorphism in the promoter region of human tissue factor pathway inhibitor (TFPI) gene does not change the plasma TFPI antigen level and does not cause venous thrombosis" THROMBOSIS AND HAEMOSTASIS , vol. 80 (2), August 1998 (1998-08), page 345-346 XP000856905 the whole document	1,4-6,10

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INTERNATIONAL SEARCH REPORT

Interr nal Application No
PCT/EP 99/06054

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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P,A	MOATTI D ET AL., : "Polymorphisms of the tissue factor pathway inhibitor (TFPI) gene in patients with acute coronary syndromes and in healthy subjects: Impact of the V264M substitution on plasma levels of TFPI." ARTERIOSCLEROSIS THROMBOSIS AND VASCULAR BIOLOGY, vol. 19 (4), April 1999 (1999-04), page 862-869 XP000856947 the whole document	1,4-6,10

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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/EP 99/06054

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9314122 A	22-07-1993	AU 675926 B	27-02-1997
		AU 3346093 A	03-08-1993
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		CZ 9401644 A	15-12-1994
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		NO 942549 A	07-09-1994
		NZ 246570 A	25-09-1996
		ZA 9300096 A	10-08-1993
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		EP 0776366 A	04-06-1997
		JP 10503375 T	31-03-1998
		US 5696088 A	09-12-1997
		US 5563123 A	08-10-1996